Environmental Health Perspectives
Vol. 92, pp. 45–52, 1991

DNA-Protein Complexes Induced by Chromate and Other Carcinogens

by Max Costa*

DNA-protein complexes induced in intact Chinese hamster ovary cells by chromate have been isolated, analyzed, and compared with those induced by *cis*-platinum, ultraviolet light, and formaldehyde. Actin has been identified as one of the major proteins complexed to DNA by chromate based upon its molecular weight, isoelectric point, positive reaction with an actin polyclonal antibody, and proteolytic mapping. Chromate and *cis*-platinum both complex proteins of similar molecular weight and isoelectric points, and these complexes can be disrupted by chelating agents and sulfhydryl reducing agents, suggesting that the metal itself is participating in binding rather than having a catalytic or indirect role (i.e., oxygen radicals). In contrast, formaldehyde complexed histones to the DNA, and these complexes were not disrupted by chelating or reducing agents. An antiserum raised to chromate-induced DNA-protein complexes reacted primarily with 97,000 kDa protein that did not silver stain. Slot blots, as well as Western blots, were used to detect formation of p97 DNA crosslinks. This protein was complexed to the DNA by all four agents studied.

Introduction

Chromate compounds are well established as human carcinogens and are potently genotoxic at a number of *in vitro* and *in vivo* end points (*1–4*). The most toxicologically active form of chromium is the hexavalent oxidation state, which at physiological pH exists as an oxyanion (*3,4*). Oxyanions, such as chromate, are actively transported into cells by the sulfate transport system, resulting in a high intracellular accumulation of chromium. The hexavalent chromium reacts with a number of reducing agents in cells, including glutathione, hydrogen peroxide, microsomal enzymes, and ribonucleotides. Chromium(VI) is eventually reduced to the kinetically inert and stable trivalent form (*5,6*). During its reduction, intermediate oxidation states of chromium are formed, and the reduction process itself, as well as the formation of these intermediate states of chromium, is thought to be important in chromium genotoxicity.

The hexavalent form of chromium has been shown to produce a variety of lesions in the DNA of mammalian cells, including single-strand breaks, alkali-labile sites, DNA-DNA, and DNA-protein crosslinks (*7–9*). Chromate is a very broad-acting genotoxic agent, as evidenced by its ability to directly induce lesions as well as to indirectly generate oxygen radicals and reactive intermediates (*6*). In fact, chromate is positive in almost every genotoxicity assay in which it has been tested.

DNA-protein complexes produced by chromium compounds have not been well studied in the past. These lesions, unlike the strand breaks and other DNA lesions that are readily repaired, are relatively persistent in the cell (*8,9*). Due to their lack of repair, DNA-protein complexes are likely to be present during DNA replication and may constitute a block to the replication machinery. Deletions which may result could be important in chromium carcinogenesis if the deleted DNA sequences code for a tumor-suppressor gene or are involved in the regulation of these important genes. The persistence of the chromate-induced DNA-protein complex makes it an interesting lesion to study as a marker for assessing chromate exposures. Due to the diversity of specific chemical agents that produce these lesions, it is likely that many different proteins can be complexed with DNA. In this regard, the specific proteins complexed with DNA and the nature of their bonding may produce a "fingerprint" from which the chemical nature of exposure might be deduced.

Neither the nature of DNA-protein complexes nor the number of agents that can cause the formation of this type of lesion has been well studied, primarily because the methodology to examine DNA-protein complexes is limited. The primary method currently in use is alkaline elution (*10*), which detects these lesions indirectly and cannot characterize their composition.

In the present study, we have examined DNA-

*Institute of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016.

protein complexes formed by chromate, formaldehyde, ultraviolet (UV) light, and *cis*-platinum, agents which have previously been shown to produce these lesions (*11*). We describe methods for isolating and characterizing these complexes with respect to the stability of the protein crosslinked to the DNA. We have also studied the specific proteins that are crosslinked to the DNA and have used immunological methods to detect the formation of DNA-protein complexes.

Methods

Chinese hamster ovary cells were treated in culture with chromate compounds as well as UV, formaldehyde, and *cis*-platinum by methods previously described (*12*). Following treatment, DNA-protein complexes were isolated from intact cells, as well as from isolated nuclei, by sodium dodecyl sulfate (SDS) lysis followed by

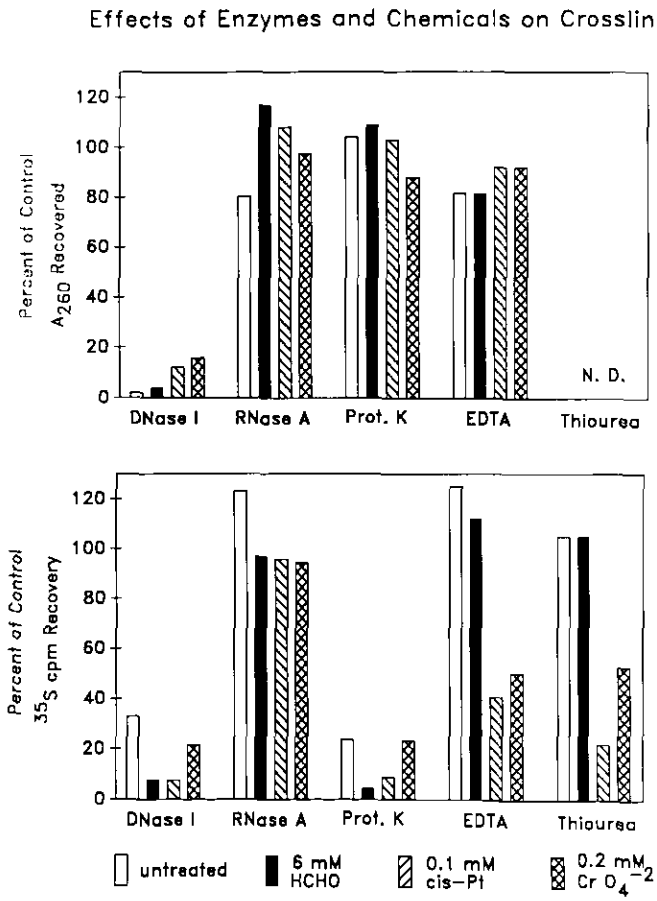

FIGURE 1. Analysis of crosslink stability with chemicals and enzymes. DNA and protein components of purified crosslinks were recovered from untreated cells and from cells exposed to 100 μM *cis*-platinum, 6 mM formaldehyde, or 200 μM potassium chromate. Samples were treated with the various enzymatic or chemical agents listed in the figure prior to centrifugation to determine whether protein or DNA recovery was sensitive to these disruptive agents. Reproduced with permission from *Molecular Toxicology* (*12*).

FIGURE 2. Nonequilibrium two-dimensional electrophoresis of DNA-protein complexes isolated from nuclei of chromate-treated cells. DNA-protein complexes were isolated from nuclei of control or potassium chromate-treated cells. (*A*) Two-dimensional gel of the control DNA-protein complexes. (*B,C*) Proteins dissociated from DNA-protein complexes generated by treating cells with 33 and 100 μM potassium chromate, respectively, prior to isolation. For each gel, 200 μg DNA was loaded. Reproduced with permission from *Molecular Carcinogenesis* (*18*).

FIGURE 3. Enzymatic digestion of DNA-protein complexes is not required for the visualization of the complexed proteins. Fifty-microgram aliquots of DNA isolated from cells treated with 200 μM potassium chromate for 24 hr were incubated in the absence or presence of nucleolytic enzymes. (*A*) Two-dimensional gel of the undigested sample. (*B,C*) Gels of the DNAse (0.2 mg/mL) and RNAse A (0.1 mg/mL) digested samples, respectively. Reproduced with permission from *Molecular Carcinogenesis* (*18*).

ultracentrifugation collection of the DNA complexed with protein, as previously described (*12–14*). The DNA-containing complexed proteins were washed extensively with SDS, urea, and high salt to dissociate noncovalently bound proteins from the DNA (*13*). The proteins covalently complexed to the DNA could be released by degrading the DNA with DNAse or, in the case of metal-induced complexes, by treatment with specific agents that are known to dissociate proteins from the DNA. The mercaptoethanol present during protein separation by electrophoresis was sufficient in the case of chromium and *cis*-platinum-induced DNA-protein complexes to cause this dissociation. In contrast, complexes formed by formaldehyde or UV light were not affected by the presence of mercaptoethanol and required treatment with DNAse in order to resolve the complexed proteins in polyacrylamide gels (*12,14*).

An antiserum to DNA-protein complexes was raised in rabbits, as previously described (*13*). Western blotting and slot blotting were also performed, as previously described (*13,14*).

Results

Figure 1 illustrates the stability of protein and DNA components of DNA-protein complexes formed by chromate, *cis*-platinum, and formaldehyde in intact Chinese hamster ovary cells. Figure 1 also characterizes the background of DNA-protein complexes that were present in untreated cells. Treatment of the DNA-protein complexes with DNAse caused decreased recovery of the DNA component in parallel with the loss of protein. This important control, which is routinely performed in these experiments, illustrates that protein recovery depends upon the integrity of the DNA. This indicates that the centrifugation recovery of protein is DNA-complex dependent and is not an artifact of altered protein solubility due to metal binding. Figure 1 also shows the effect of RNAse treatment, which did not significantly reduce recovery of either component of the complex. Protease K, however, eliminated the protein recovery.

As expected, EDTA treatment did not affect recovery of DNA; however, with metal-induced complexes, the recovery of the protein component was substantially reduced by EDTA (Fig. 1). In contrast, recovery of this protein component from protein-DNA complex formed by formaldehyde or those present in the background of untreated cells were not affected by EDTA. Similarly to EDTA, thiourea also dissociated the protein-DNA complexes induced by metals, but not those present in untreated cells or those formed by formaldehyde. These results illustrate that a substantial portion of the DNA-protein complexes formed by *cis*-platinum or chromium involved direct participation of the metals in the complex formation. Additionally, the effect of EDTA on chromate complexes suggests that the trivalent form of chromium is involved in the DNA-protein complex, since this is the only form of chromium that can be chelated by EDTA.

Figure 2 examines the protein components of the DNA-protein complex induced by chromate. Figure 2*A* shows the absence of proteins associated with 200 μg of DNA from untreated cells. Following treatment with chromate at 33 μM, there were three major proteins

FIGURE 4. Two-dimensional gel electrophoresis of nuclear protein lysate and formaldehyde-induced crosslinks. (*A*) Silver-stained, nonequilibrium two-dimensional gel of 40 μg of a nuclear protein lysate run for approximately 1200 V/hr. The *H* denotes the position of the core histones. (*B*) Silver-stained gel following electrophoresis of two A_{260} units of crosslink material from untreated cells; no proteins were detectable under these conditions or when control material was digested with DNAse I before electrophoresis (not shown). (*C,D*) DNA-protein crosslinks isolated from cells treated with 13 mM HCHO for 1.5 hr. The gel in (*C*) shows no protein has entered the gel, while histones are visible in (*D*) (labeled *H*). The crosslinks in (*D*) were digested with DNAse I prior to electrophoresis. Contaminating DNAse I proteins are indicated by *d*. Reproduced with permission from *Molecular Toxicology* (*12*).

FIGURE 5. Two-dimensional gel analysis and immunoblotting of nuclear proteins to locate the 95-kDa antigen. (*A*) Silver-stained two-dimensional gel of a control nuclear protein fraction (25 μg protein). The 95-kDa protein is indicated as the unstained area. Actin is also indicated for purposes of comparison. (*B*) Radiograph of a duplicate blot of the gel in (*A*) reacted with the DNA-protein complex antiserum and ^{125}I protein A. Reproduced with permission from *Carcinogenesis* (*13*).

complexed with DNA. Enhanced levels of these same proteins, plus a number of additional proteins, were found following 100 μM chromium treatment (Fig. 2*C*).

Figure 3 illustrates that the proteins complexed to DNA by chromate were released during electrophoresis without prior treatment with DNAse or RNAse. Degradation of DNA did not enhance the number of proteins that were resolved in the gels, suggesting that the conditions of electrophoresis, as well as the presence of mercaptoethanol during electrophoresis, were sufficient to release the complexed protein from the DNA. *cis*-Platinum complexed proteins of similar molecular weight and isoelectric point to those found with chromate.

Figure 4 examines the proteins that were complexed to the DNA by formaldehyde. In Figure 4*A*, a nuclear protein lysate analyzed by two-dimensional gel electrophoresis shows the location of histone proteins. Figure 4*B* illustrates a silver-stained gel used to analyze crosslinked material from untreated cells. No proteins were be detected in untreated cells with the DNA load that was utilized in this gel. Figures 4*C* and 4*D* shows the DNA-protein crosslinks at a similar load of DNA from cells treated with formaldehyde. In Figure 4*C*, DNA-protein complexes were not treated with DNAse prior to analysis on the gel, whereas in Figure 4*D* the DNA was previously degraded. Figure 4*D* demonstrates that histones were the predominant proteins that were complexed to the DNA by formaldehyde. The spots identified in the figure by the letter *d* are those proteins that were introduced by the addition of DNAse enzyme.

One of the major proteins complexed to the DNA by *cis*-platinum and chromate was a 45,000-Da protein with the same molecular weight and isoelectric point as actin. In fact, a purified actin standard migrates to the same location in the gels as this p45 protein. We have recently confirmed the identity of this protein as actin by its positive reaction with an actin polyclonal antibody and by V8 protease mapping (data not shown).

Another approach taken to study DNA-protein complexes uses an antiserum raised by rabbits against the chromate-induced DNA-protein complexes formed in Chinese hamster ovary cells (*13*). Figure 5 illustrates the reactivity of this antiserum toward a Chinese hamster nuclear protein lysate. Figure 5*A* shows the silver staining of the nuclear protein lysate, while Figure 5*B* is a Western blot reacted with the DNA-protein complexed antiserum and ^{125}I protein A. As depicted in Figure 5*B*, the antibody recognized a 95,000-Da protein, which is not silver stained, in the left-hand side of Figure 5*A*. Recently, more accurate molecular weight determinations of this protein confirm a 97,000 Da molecular weight. The lack of silver staining of this protein, however, precluded its detection when the proteins of chromate-induced DNA-protein complexes were initially studied (Figs. 2, 3). Figure 6 illustrates a one-dimensional gel of DNA-protein complexes induced by chromate in Chinese hamster ovary cells whose proteins were radiolabeled with ^{35}S-methionine prior to chromate treatment. In this Figure 6, a ^{35}S-methionine radiolabeled protein is seen complexed to the DNA in the molecular weight range of p97.

FIGURE 6. ^{35}S fluorography of proteins complexed to DNA after chromate treatment for 24 hr. DNA was extracted with SDS and urea, digested with DNAse I (5 μg/mL), lyophilized, and loaded as 100 μg DNA/lane. The dried gel was exposed to film to detect the labeled proteins. The concentrations of chromate treatment are indicated above each lane. Reproduced with permission from *Carcinogenesis* (*13*).

Figure 7 illustrates the reactivity of the DNA-protein complex antibody toward complexes induced by agents other than chromate, including *cis*-platinum, formaldehyde, and UV light. In the left lanes, the DNA-protein complexes were not treated with DNAse prior to gel loading. In the absence of DNAse, only the proteins complexed with *cis*-platinum were able to enter the gel and react with the antiserum. In contrast, the right-hand lanes contain complexes that were treated with DNAse prior to electrophoresis. Formaldehyde and UV-induced complexes can be resolved by this treatment to show the antiserum reaction with the p97 protein which was complexed to the DNA. Figure 8 shows that proteins from chromate-induced complexes could readily be released into the gel without DNAse treatment and are detectable on the gel with the antiserum as expected.

Figure 9 illustrates the use of the same DNA-protein complex antibody to detect complexes formed by various agents in a slot-blot protocol. In Figure 9, the antiserum reacted with DNA-protein complexes from cells treated with *cis*-platinum, chromate, and formaldehyde. Figure 9 illustrates antibody detection of those complexes formed by all three chemical agents, including untreated cellular DNA at high DNA loading, but not at the lower level of 5 μg DNA. As previously noted in Figure 8, the antiserum reacted primarily with the protein component of the complex. This is supported by Figure 9, which shows protease K eliminated the antibody reaction. In contrast, treatment with DNAse did not substantially affect the reactivity of this antibody, although there was a slight reduction in the background reactivity of untreated cells.

Discussion

DNA-protein complexes formed by metals have previously been shown to involve the direct participation of the metal in the complex (*12*). In the case of chromate, the trivalent form of chromium was implicated in the formation of these complexes (*12*). One possible mechanism for complex formation involves the trivalent chromium-mediated reaction of the N7 position of guanine with a sulfur of the amino acid cysteine. This model is derived from data regarding the sensitivity of the complex to sulfhydryl reducing reagents, the effects of EDTA on the complex, and the binding constants of chromium for various ligands.

Chromate and *cis*-platinum DNA-protein complexes were very similar in their protein composition, since two-dimensional gels separating these proteins are virtually indistinguishable. One of these proteins has been positively identified as actin, by virtue of its identical molecular weight and isoelectric point as actin, the reactivity of the p45 protein from chromate-induced complexes with an actin polyclonal antibody, and the proteolytic map of this protein, which was similar to that of bovine muscle and human platelet actin (data not shown).

Histones were complexed to the DNA by formaldehyde, but were not complexed by the other agents examined in this study. This is probably due to the high reactivity of formaldehyde at the ε-amino group of lysine. The proteins complexed to the DNA by chromate and *cis*-platinum are similar in molecular weight and isoelectric points to those proteins which are present at very high concentration in the nuclear matrix. Association of DNA with the nuclear matrix is known to be important in DNA repair and transcription. Actin, which is known to be an important component of transcriptional complexes and is involved in DNA repair, is also an abundant protein in the nuclear matrix (*15,16*). The metal-induced complexing of actin to the DNA is of interest in light of recent studies demonstrating that chromate can selectively inhibit inducible gene expression (*17*).

FIGURE 7. Comparison of the p97-DNA interactions induced by different crosslinking agents using Western blots and antiserum detection. The purified crosslink materials were run in a 12.5% SDS gel under reducing conditions. Prior to electrophoresis and blotting, some samples were digested with DNAse I as indicated. p97 was detected with antiserum and ^{125}I-protein A followed by autogradiography. Reproduced with permission from *Mutation Research* (*14*).

FIGURE 9. Slot blot detection of p97 crosslinks formed in cultured cells using p97 antiserum. Chinese hamster ovary cells were treated with metals or formaldehyde and crosslinked material was isolated as described in the methods. DNA loads were bound to the nitrocellulose sheets (in triplicate) and antigen detection was achieved with antiserum and ^{125}I-protein A. In some instances the complexes were digested with DNAse I or proteinase K prior to their application to the filter to assess the nature of the antigenic reactivity. Reproduced with permission from *Mutation Research* (*14*).

The rabbit antiserum raised to chromate-induced DNA-protein complexes reacted primarily with a protein that was not initially detected on the silver-stained gels in which the DNA-complexed proteins were analyzed. This antiserum reacted with a 97,000 Da protein that was apparently complexed to the DNA by formaldehyde, *cis*-platinum, UV light, and chromate. This protein is very interesting for future studies due to its ability to be complexed with DNA by a variety of chemical agents and due to its lack of silver staining. The antiserum to p97 has also been used to detect the formation of DNA-protein complexes by various chemical agents on slot blots (*14*). This is a simple method which appears to be useful in monitoring the formation of these complexes. Currently, these methods are being applied to the detection of chromate-induced DNA-protein crosslinks formed *in vivo*, which may be sensitive biomarkers of chromate exposure.

FIGURE 8. Influence of 2-mercaptoethanol on DNA-protein complex interaction. Undigested DNA-protein complexes (250 μg DNA/lane) were run on a one-dimensional gel and blotted to nitrocellulose in the absence (lanes 1,2) or presence of 2-mercaptoethanol (lanes 3–6). The autoradiograph of the blot probed with anti-complex serum and ^{125}I-protein A is shown in lanes 1–4. Lanes 5 and 6 were stained with amido black. Lanes marked (–) are from untreated cells and lanes marked (+) are from chromate-treated cells (200 μM, 24 hr). Reproduced with permission from *Carcinogenesis* (*13*).

This work was supported by grants ES 04715 and ES 04895 from the National Institute of Environmental Health Sciences and by grant R814702 from the U.S. Environmental Protection Agency. The author would like to thank Charles Miller for performing most of the experiments discussed in this manuscript. The secretarial assistance of Mary Bader is gratefully acknowledged. The author also thanks Catherine Kleine and Mitchell Cohen for their review of this manuscript.

REFERENCES

1. Sunderman, F. W. Carcinogenicity and mutagenicity of some metals and their compounds. In: IARC Monographs: Environmental Carcinogens—Selected Methods of Analysis (I. K. O'Neill, P. Schuller, and L. Fishbein, Eds.), Oxford University Press, Oxford, England, 1986, pp. 17–43.
2. Hertel, R. F. Sources of exposure and biological effects of chromium. In: IARC Monographs: Environmental Carcinogens—Selected Methods of Analysis (I. K. O'Neill, P. Schuller, and I. Fishbein, Eds.), Oxford University Press, Oxford, England, 1986, pp. 63–77.
3. Levis, A. G., and Bianchi, V. Mutagenic and cytogenetic effects of chromium compounds. In: Biological and Environmental Aspects of Chromium (S. Langard, Ed.), Elsevier Biomedical Press, Amsterdam, 1982, pp. 171–208.

4. Heck, J.D., and Costa, M. In vitro assessment of the toxicity of metal compounds. I. Mammalian cell transformation. Biol. Trace Elem. Res. 4: 71–82 (1982).
5. Arslan, P., Beltrame, M., and Tomasi, A. Intracellular chromium reduction. Biochim. Biophys. Acta 931: 10–15 (1987).
6. Connett, P. H., and Wetterhahn, K. Metabolism of the carcinogen chromate by cellular constituents. Structure Bond. 54: 93–124 (1983).
7. Tsapakos, M. J., Hampton, T. H., and Wetterhahn, K. E. Chromium(VI)-induced DNA lesions and chromium distribution in rat kidney, liver, and lung. Cancer Res. 43: 5662-5667 (1983).
8. Sugiyama, M., Wang, X. W., and Costa, M. Comparison of DNA lesions and cytotoxicity induced by calcium chromate in human, mouse, and hamster cell lines. Cancer Res. 46: 4547–4551 (1986).
9. Sugiyama, M., Patierno, S. R., Cantoni, O., and Costa, M. Characterization of DNA lesions induced by $CaCrO_4$ in synchronous and asynchronous cultured mammalian cells. Mol. Pharmacol. 29: 606–613 (1986).
10. Kohn, K. W., Ewig, R. A. G., Erickson, K. C., and Zwelling, L. A. Measurement of strand breaks and crosslinks by alkaline elution. In: DNA Repair: A Laboratory Manual of Research Procedures, Vol. 1, Part B (E. Freidberg and P. H. Hanawalt, Eds.), Marcel Dekker, New York, 1981, pp. 379–401.
11. Oleinick, N. L., Chiu, S., Ramakrishnan, N., and Xue, L. The formation, identification, and significance of DNA-protein crosslinks in mammalian cells. Br. J. Cancer 55: 135–140 (1987).
12. Miller, C. A., III, and Costa, M. Analysis of proteins crosslinked to DNA after treatment of cells with formaldehyde, chromate, and *cis*-diamminedichloroplatinum(II). Mol. Toxicol. 2: 11–26 (1989).
13. Miller, C. A., III, and Costa, M. Immunological detection of DNA-protein complexes induced by chromate. Carcinogenesis 10(4): 667–672 (1989).
14. Miller, C. A., III, and Costa, M. Immunodetection of DNA-protein crosslinks by slot blotting. Mutat. Res. 234: 97–106 (1990).
15. Pienta, K. J., Partin, A. W., and Coffey, D. S. Cancer as a disease of DNA organization and dynamic cell structure. Cancer Res. 49: 2525–2532 (1989).
16. Eastman, A. Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro (ethylenediammine) platinum(II). Biochemistry 22: 3927–3933 (1983).
17. Hamilton, J. W., and Wetterhahn, K. E. Differential effects of chromium(VI) on constitutive and inducible gene expression in chick embryo liver in vivo and correlation with chromium(VI)-induced DNA damage. Mol. Carcinog. 2: 274–286 (1989).
18. Miller, C. A., III, and Costa, M. Characterization of DNA protein complexes induced in intact cells by the carcinogen chromate. Mol. Carcinog. 1: 125–133 (1988).